Amperometric enzyme-based biosensors: refined bioanalytical tools for in vivo biomonitoring

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CHAPTER 5

The impact of sterilization on the performance of an implantable enzyme-based glucose biosensor.

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Abstract:

Although several biosensors have been developed for continuous *in vivo* monitoring of glucose, only a few devices are routinely used. The lesser biosensor performance after a sterilizing treatment, a requirement for any implantable biomedical device, is probably one of the reasons for the limited clinical application of glucose biosensors.

Here, we describe the effect of sterilization on the performance of flexible implantable amperometric enzyme-based biosensors for subcutaneous *in vivo* real-time monitoring of glucose. *In vitro* electrochemical characterization revealed that these biosensors were highly selective, and sensitive (LRS ≥ 70 nA/μM) in the relevant physiological range (LR ≥ 14 mM). Additionally, we have thoroughly evaluated the impact of sterilization on the performance of this biosensor, both directly after sterilization and during a 4 week storage period. Four different methods for sterilization were applied: treatment with ethylene oxide i), treatment with H_2O_2, alone ii) or combined with gamma-radiation iii), and finally treatment with a mixture of chlorohexidine and isopropyl alcohol iv). Sterilized biosensors were evaluated and thoroughly characterized *in vitro*.

Our data showed an immediate and negative influence of sterilization on biosensor performance. Additionally, we observed that for some sterilization methods, the biosensors retained adequate for implantation, even 4 weeks after being exposed to those methods.

Keywords: *In vivo*, implantable biosensors, shelf-life, sterilization, glucose

5.1-Introduction

Nowadays, biosensors can be based on a myriad of transducers coupled to a multitude of biorecognition elements, (Hammond et al. 2016; Turner 2013; Vigneshvar et al. 2016). However, only a limited amount of the many biosensors described in the literature are routinely employed (Siontorou and Batzias 2010). Nevertheless, there is a significant number of biosensors successfully employed in *in vivo* biomonitoring (Hamdan and Mohd Zain 2014; Wilson and Ammam 2007; Wilson and Gifford 2005).

Amperometric enzyme-based ones are arguably the most successful type of biosensors, and have been even effective in *in vivo* brain biomonitoring of a series of key biomarkers including neurotransmitters (Ahmad et al. 2008; Burmeister et al. 2002; Calia et al. 2009; Cordeiro et al. 2015; Pomerleau et al. 2003; Sirca et al. 2014; Vasylieva et al. 2011; Wahono et al. 2012). Nevertheless, most of its applications still focus on *in vivo* monitoring of glucose (Koschwanez and Reichert 2007; Vaddiraju et al. 2010; Wilson and Gifford 2005). Initially, these type of biosensors were designed for implantation in blood vessels (Kondo et al. 1982), in order to replace the gold-standard methods in glucose biomonitoring. However, poor in vivo performance redefined the target implantation site and nowadays, implantable biosensors for *in vivo* glucose biomonitoring are applied to subcutaneous implantation (Yoo and Lee 2010).

Although several implantable biosensors for *in vivo* glucose biomonitoring are now available (Rodbard 2016), a series of issues is still hampering their widespread application as replacement of the conventional glucose biomonitoring methods (Keenan et al. 2009; Kovatchev et al. 2008; Lodwig and Heinemann 2003; Pickup et al. 2005; Vaddiraju et al. 2010).

Optimization of implantable amperometric enzyme-based biosensors focuses on higher accuracy and precision, improved biocompatibility and low cytotoxicity. The goal is, to reliably monitor glucose for as long as possible and eventually to reach the “Holy Grail”, a closed-loop device with a glucose monitoring system coupled to an automated insulin administration device (Aye et al. 2010; Facchinetti et al. 2010; Mastrototaro and Lee 2009; Penfornis et al. 2011; Wang 2008).

Although largely neglected, sterilization is a requirement for optimized implantable enzyme-based biosensors (Oberländer et al. 2015; von Woedtke et al. 2002). However an efficient sterilization method has to ensure not only the needs of sterility assurance but has also to guarantee the functionality of the sterilized product. Note that the same active principles that damage vital microorganism are able to influence the materials, as well as the functionality of the biosensors (von Woedtke and Kramer 2008).

For sterilization procedures, the pharmacopeias demands a sterilization assurance level (SAL) of 10^{-6}, i.e. a probability of not more than one viable microorganism in $1x10^{6}$ sterilized items. Sterilization by ethylene oxide gas or by ionizing radiation, such as γ radiation, are amongst the recommended methods from pharmacopeias (Commision 2016; Europe 2014).

However, these methods usually compromise the functionality of the products and are often regarded as harsh. Therefore milder variations, such as the combination of H_2O_2 with low intensity γ-radiation have been explored (von Woedtke et al. 2002; von Woedtke and Kramer 2008).

An alternative to terminal sterilization is aseptic processing (Daniell et al. 2016). However, aseptic processing is less cost-effective when compared with terminal sterilization. Additionally, aseptic processing has been reported as less safe than terminal sterilization procedures (von Woedtke and Kramer 2008).

Although the guidelines for SAL are quite strict, some authors advocate a tiered SAL table, adjusted according to the respective utilization task. According to that alternative view a SAL 10^{-6} for heat-resistant would have a suggested term: "Pharmaceutical sterilization", while SAL a 10^{-4} would be considered "High level sterilization". Methods that would ensure SAL 10^{-3} for heat-sensitive re-usable medical devices, would be considered "Low-level sterilization"(von Woedtke and Kramer 2008).

Nonetheless, only a few dozen studies have been devoted to the impact of sterilization on biosensor performance and were thoroughly reviewed by von Woedtke (von Woedtke et al. 2002; von Woedtke and Kramer 2008). Most of these studies show a significant impairment of biosensor performance after sterilization. Unfortunately most of these studies focused on the acute effects of sterilization and consider only sensitivity as the sole biosensor performance parameter.

Here we describe and characterize, the acute and long-term effects of sterilization on a novel flexible implantable amperometric enzyme-based biosensor for *in vivo* real-time continuous glucose monitoring in subcutaneous tissue. Our biosensor includes a flexible lead, coupled to a needle type microelectrode that can easily be incorporated in a CGM device. The microelectrode surface was functionalized with the permselective membrane Nafion. Although not entirely selective, Nafion is biocompatible, and dramatically increases biosensor selectivity towards the major non- specific electroactive species present in subcutaneous tissue: uric acid (UA) and ascorbic acid (AA)(Cordeiro et al. 2016; Oldenziel and Westerink 2005; Sirca et al. 2014; Wahono et al. 2012; Walker et al. 2007). The specific enzyme, (Glucose Oxidase) was immobilized in an hydrogel, assembled onto microelectrodes previously functionalized with Nafion. In addition, to prevent, at least partially, the deleterious effects of biofouling, (Jusoh et al. 2012; Koh et al. 2011; Ward 2007) we covered the biosensor with a hollow regenerated cellulose based dialysis membranes (Figure 1).

In order to evaluate, the impact of sterilization on the performance of the biosensors we sterilized them with a series of methods that assured proper SAL. Sterilized biosensors were then electrochemically evaluated *in vitro*. Based on *in vitro* calibrations we were able to estimate a series of relevant biosensor bioanalytical properties, both acutely and up to 4 weeks storage after each sterilization procedure.

5.2-Materials and Methods

5.2.1- Materials

Glucose Oxidase (Asperillus Niger , Type-XII , 100kU), Nafion (25% v/v in aliphatic alcohols), D-Glucose, uric acid (UA), l-ascorbic acid (AA) were obtained from Sigma (St. Louis, Missouri, USA) without further purification, H_2O_2 (35 % v/w), Bovine Serum Albumin, Glutheraldehyde (GA) (grade I, 25%, aqueous solution) were purchased from Sigma Aldrich(St. Louis, Missouri, USA). PtIr wire (125 µm Ø) was obtained from Advent Research Materials (Oxford, UK). Chlorohexidine was purchased from VWR (The Netherlands). Regenerated Cellulose (RC) membranes were obtained from Brainlink BV (Groningen, The Netherlands). The prototype flexible leads were kindly provided from Data Sciences International (DSI) (St Paul, Minneapolis, USA).

A phosphate buffer solution (PBS) was used containing 145 mM Na^+, 1.2 mM $Ca2^+$, 2.7 mM K^+, 1.0 mM $Mg2^+$, 152 mm Cl^-, and 2.0 mM PO^{4-} in ultrapurified water, brought to pH 7.4 with sodium hydroxide and degassed before use.

5.2.2- Biosensor assembly

The implantable glucose biosensors (Figure 1) were assembled based on a previously described protocol (Cordeiro et al. 2015). Briefly, the surface of Pt/Ir needle type electrodes (125 µm Ø x 10 mm) was modified on “layer by layer” manner. A permselective membrane (Nafion) was assembled on the surface of bare electrodes. Then, Nafion coated microelectrodes were manually coated (25 x) with an enzymatic hydrogel containing BSA (1%), Glutheraldehyde (0.125%) and Glucose Oxidase (0.2 U/µL) and allowed to cure for at least 48 hours. Finally, a hollow microdialysis membrane (RC) was applied onto the microbiosensors.

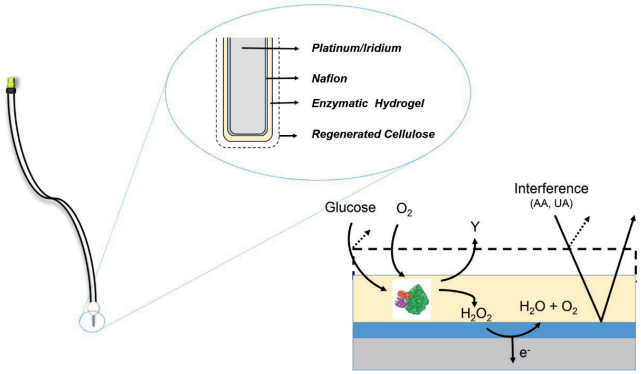

Figure 1- A- Schematic representation of a fully assembled flexible implantable amperometric enzyme-based biosensors for *in vivo* glucose biomonitoring. B-Molecular geometry and working mechanism of the amperometric enzyme-based biosensor.

5.2.3- Sterilization procedures

All biosensors, except the ones in the control group, were sterilized by a method that would enable at least a 10^{-4}SAL (von Woedtke et al. 2002). The procedures for sterilization are as described.

5.2.3.1- Ethylene oxide

Biosensors were sterilized in validated EtOx gas sterilizers (Data Sciences International, Minneapolis, USA) using a three phase method; Pre-conditioning, Sterilization: and Aeration (to contain and eliminate residual EO emissions to ensure that it meets specified residual limits outlined in ISO 10993-7).

5.2.3.2- γ- Radiation + H_2O_2

For γ-irradiation, Cesium 137 radioisotope sources (FWM, Radiotherapy, Groningen, The Netherlands) were employed. The absorbed radiation was controlled, based on dosimetry and the time of exposure. Biosensors were exposed to a total amount of radiation of 9 kGy. After, biosensors were immersed in an hydrogen peroxide solution (0.8%) for 4 days (von Woedtke et al. 2002).

5.2.3.3- Clorohexidine combined with Isopropyl Alcohol (IPA)

To evaluate the impact of a sterilization with IPA/chlorohexidine, biosensors were

immersed in a solution of IPA (70% V/V) containing 5% (w/V) of chlorohexidine gluconate for 30 minutes (Ahmed et al. 2000).

5.2.3.4- Hydrogen Peroxide

To test sterilization with hydrogen peroxide, biosensors were immersed in a 0.8% H_2O_2 solution for 4 days.

5.2.4-Electrochemical evaluation

In vitro microelectrode evaluations were carried as previously described (Cordeiro et al. 2015). Biosensor were immersed in a constantly stirred PBS (pH 7.4) at 37º C, with an applied potential of 700 mV vs. Ag/AgCl using a potentiostat (Pinnacle, model 3104 Pinnacle Tech. Inc., USA). After an initial equilibration period (approximately 45 min) when a stable current was reached steady state parameters (noise and baseline) were assessed. Then, non-specific electroactive species (UA 200 µM; and AA 250 µM) were added sequentially, prior to consecutive additions of Glucose (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 8, 16 and 32 mM) and H_2O_2 (50, 100 and 200 µM) (Burmeister et al. 2002; Wahono et al. 2012; Walker et al. 2007). We monitored changes in oxidation currents in response to analyte addition. Electrochemical evaluation was performed prior and after each sterilization procedure, when applicable. Additional evaluations were performed weekly, up to 4 weeks after sterilization to all biosensors.

5.2.5-Data analysis and statistical evaluation

All biosensor performance parameters were estimated based on the observed changes in oxidation currents. Noise and limit of detection (LOD) were calculated by linear regression, whereas linear range (LR), linear range slope (LRS), apparent Michaelis-Menten constant ($_{app}K_M$) and maximum current intensity (I_{Max}) were calculated using non-linear regression, using a Michaelis-Menten derived kinetic model(O'Neill et al. 2008). Selectivity coefficients (SC) were calculated as previously described (Cordeiro et al. 2016). Linear and non-linear regression analysis were performed using GraphPad Prism 5.0. All data are presented as mean±SEM (standard error of the mean).

All parameters were statistically evaluated either by one-way (PreC vs PostC) or two-way ANOVA (long term evaluation). When necessary, additional Bonferroni post- hoc tests were performed and $p \leq 0.05$ and $p \leq 0.001$ was considered, statistically significant and highly significant, respectively. All statistical analyses were performed using SigmaStat 12.0.

5.3- Results and Discussion

To understand the impact of sterilization on the performance of implantable amperometric enzyme-based biosensors, we compared a range of performance parameters, prior and after sterilization.

Most, if not all of the few studies devoted to the effect of sterilization on biosensor performance, are focused on immediate effects. Moreover, all of them only evaluate a limited amount of parameters (von Woedtke et al. 2002). In this study, we have evaluated, a much larger range of key biosensors parameters. Furthermore, we evaluated not only the acute effect of sterilization procedure, but also its impact on a longer periods (up to 4 weeks). Additionally, we assessed not only the impact of each sterilization procedure on biosensor sensitivity towards glucose, but also its effect on biosensor surface availability (by monitoring responses to H_2O_2) and biosensor selectivity (by monitoring the SC for both AA and UA).

5.3.1- Pre Sterilization evaluation

In vitro evaluation of biosensor performance revealed that a steady-state of the sensor current was consistently achieved 45 to 60 min after its immersion in the buffer (Figure 2).

Our data show that these biosensors take at least 15 additional minutes more to stabilize when compared with biosensors with the same diameter but shorter length (1 mm)(Cordeiro et al. 2015). It seems that *in vitro* stabilization time is dependent on the total electrode surface. The use of an outer membrane (and its need for some hydration time) may also play a role in the increase of the time necessary to reach steady-state, previously described (Baronas and Kulys 2008).

Based on the steady state currents we estimated the noise levels of the biosensors prior to the sterilization. We observed low noise levels ($\leq$ 100 pA) for the described implantable biosensor (Table 1 A).

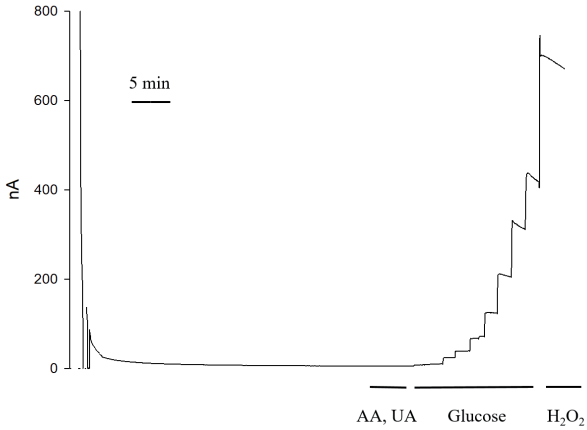

Figure 2- Typical *in vitro* calibration of a non-sterilized implantable glucose biosensor. Upon establishing a stable baseline current we monitored changes in oxidation current in response to the consecutive administration of intereferants (AA and UA) and target analytes (Glucose (0-32 mM and H_2O_2 – 200 μM).

In vitro calibrations revealed that these biosensors were selective by against the main non-specific electroactive species present in subcutaneous interstitial tissue (AA and UA) in its relevant range. Electrochemical evaluation showed that the developed biosensors were highly sensitive to both Glucose and H_2O_2 (Figure 2). Based on *in vitro* calibrations we have plotted the changes in oxidation current versus the levels of glucose (Figure 3).

We observed a high linear correlation between glucose and the measured oxidation currents throughout the whole calibration range ($R^2 \geq 0.9$). However, there was an apparent decrease in linearity for high glucose levels (≥ 16 mM), consistent with a typical Michalis-Menten-like behavior (Rothwell et al. 2010).

We fitted the data form the calibration with a non-linear kinetic model (Cordeiro et al. 2015; O'Neill et al. 2008), for enzymes immobilized in electrochemical biosensors and its derivation for surface independent kinetics. The results show a nearly perfect fit ($R^2 \geq 0.99$) and allowed us to estimate several key biosensor parameters.

Our data show a low LOD for both glucose and H_2O_2 (2.96 ± 0.05 and 0.23±0.05 μM respectively), indicating its suitability for monitoring real-time subcutaneous glucose levels. Additionally we estimated a high $_{app}K_M$ (28.12 ±0.78 mM), which is not far from the enzyme activity in aqueous solution (33 mM) (Szajáni et al. 1987). The use of a crosslinking agent such as GA, in the hydrogel assembly results in a decrease in $_{app}K_M$, thereby increasing biosensor affinity. However the use of an outer membrane decreased the biosensor affinity, probably by affecting glucose diffusion rate (Vasylieva et al. 2011). Microdialysis membranes, such as RC, were described as able to increase the LR by reducing the analyte diffusion rates (Cordeiro et al. 2015). Accordingly, the high LR (14.06 ±0.39 mM) observed, is most likely

due to the decrease in diffusion rate, compensating for the increase in affinity due to enzyme immobilization. Nevertheless, all biosensors were highly sensitive for both glucose and H_2O_2 (LRS of 77.67±0.02 nA/mM for glucose and H_2O_2 1.91±0.18 nA/μM). The sensitivity towards glucose is within the same range or even higher than some of reportedly highly sensitive glucose biosensors (Hossain and Park 2016; Qiang et al. 2011; Vaddiraju et al. 2013), that were successfully employed for *in vivo* glucose biomonitoring. Our data suggest, that the presently described biosensors may be able to reliably monitor not only basal values, but also to monitor changes in glucose levels in subcutaneous tissue.

The use of a non-linear model for kinetic analysis (O'Neill et al. 2008) combined with the use of mathematical models for selectivity (Cordeiro et al. 2016) allowed us to estimate 14 relevant biosensor performance parameters. This number, allowed us to study the effects of sterilization on biosensor performance, in great detail.

5.3.2- Post Calibration - Short term

All biosensors were re-evaluated *in vitro* immediately after each sterilization procedure. The data obtained from the post calibrations (PostC) were then compared to data determined prior to sterilization. Post-calibrations clearly show (Figure 3) that sterilization, regardless of the method, results in a significant decrease (at least 2 fold; $p< 0.05$) in oxidation currents. In addition we found significant differences in oxidation currents, hence biosensor performance parameters, amongst the various tested sterilization procedures (Table 1 A and B).

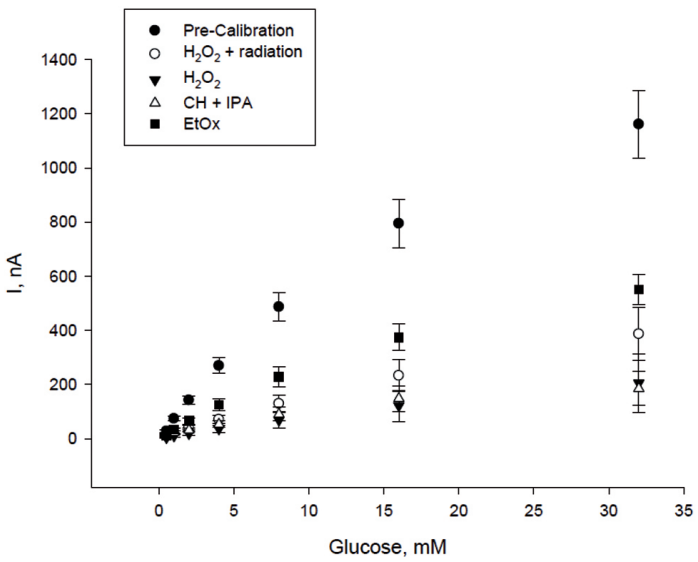

Figure 3 - *In vitro* calibration of the sensors prior and after each sterilization procedure. Data are mean+SEM.

Using the data from the post-calibration we estimated the performance parameters of the biosensors after sterilization (Table 1 A and B). The decrease in oxidation current due to biosensor sterilization culminated in significant changes in glucose and H_2O_2 derived biosensor performance parameters (Figure 3).

Table 1- Biosensor performance parameters estimated from the calibrations performed prior and after each sterilization method. **A**-Glucose related parameters **B**-H_2O_2 related parameters. Data are mean±SEM.

A

		Pre-Calibration n=39	γ-radiation + H_2O_2 n=8	H_2O_2 n=7	CH+IPA n=8	Ethylene Oxide n=6
Glucose	Noise (nA)	0.08±0.001	0,27 ± 0,06 **	0,07 ± 0,01	0,03 ± 0,01	0,11 ± 0,02
	LOD (µM)	2,96 ± 0,05	43,75 ± 2,77 **	21,83 ± 0,67 **	4,44 ± 0,29	9,15 ± 0,16 **
	$_{app}K_M$ (mM)	28.12 ±0.78	58,42 ± 5,21 **	64,92 ± 2,76 **	15,56 ± 2,1 **	29.40 ± 0,89
	I_{Max} (nA)	2184±34.94	1091 ± 69,24 **	621,6 ± 19,28 **	278,80 ± 17,99 **	1059 ± 18,76 **
	LRS(nA/mM)	77.67±0.02	18,68 ± 1,19 **	9,57 ± 0,30 **	17,92 ± 1,16 **	36,02 ± 0,64 **
	LR (mM)	14.06 ±0.39	29,21 ± 2,61 **	32,46 ± 1,38 **	7,78 ± 1,05 **	14,70 ± 0,44
	SI $_{app}K_M$	28.69±0.67	54,52 ± 4,53 **	59,39 ± 4,03 **	12,27 ± 1,86 **	30,01 ± 0,78
	SI I_{Max}	1.14±0.02	0,43 ± 0,03 **	0,23 ± 0,01 **	1,35 ± 0,09 **	0,36 ± 0,01 **
	SC AA	165.44±30.28	1561,41 ± 405,57 *	2915,68 ± 1059,51**	2061,36 ± 869,07 **	448,68 ± 150,22
	SC UA	128.27±22.28	960,24 ± 243,03 *	960,43 ± 276,47*	1227,44 ± 519,92 **	146,75 ± 41,18
	R^2	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99

B

		Pre-Calibration n=39	γ-radiation + H_2O_2 n=8	H_2O_2 n=7	CH+IPA n=8	Ethylene Oxide n=6
H_2O_2	LOD (µM)	0.23±0.05	0,59 ± 0,12 *	0,23 ± 0,08	0,54 ± 0,21	0,22 ± 0,06
	LRS (nA/(µM)	1.91±0.18	2,39 ± 0,19	1,89 ± 0,40	0,25 ± 0,09 **	2,96 ± 0,64 **
	SC AA	3.76±0.64	6,71 ± 1,01	2,23 ± 1,13	34,99 ± 10,24 **	6,58 ± 3,07
	SC UA	3.35±0.47	4,84 ± 0,81	3,80 ± 1,20	50,61 ± 15,19**	2,47 ± 0,39

Although all sterilization methods induced significant changes in several parameters, we observed pronounced differences between the various methods.

The LRS for H_2O_2 was altered in half of the methods tested. Only the noise level was altered in less than half of the sterilization methods. All other glucose derived parameters (9/10) were significantly different in 3 of the 4 methods tested. Both I_{Max} and glucose LRS were different when compared with PreC values for all sterilized biosensors.

Our data shows that glucose-derived parameters were more affected by sterilization, than H_2O_2 derived ones. The "layer by layer" assembly, with an additional outer membrane, may have protected the inner permselective membrane. Being on top of the Nafion membrane, the enzymatic hydrogel may be more prone to the noxious effect of the sterilization procedures. It may even act as protective barrier to the inner membrane.

Although our results clearly show that all sterilization methods induce a significant decrease in biosensor performance, it is not immediately clear to conclude which is the best one.

However, analyzing the number of performance parameters changed as measure, a rather objective conclusion can be reached (Figure 4). Sterilization by EtOx induced less changes in biosensor performance parameters (5/14) than any other method. This was apparently the milder sterilization procedure. Sterilization with either CH-IPA or γ radiation combined with H_2O_2 resulted in changes in nearly all of the estimated performance parameters (11/14). However, in the case of γ-rad, both SC for glucose displayed lower p values, thus less dramatic changes. In that sense, we can say that CH- IPA was the most acutely harmful sterilization method.

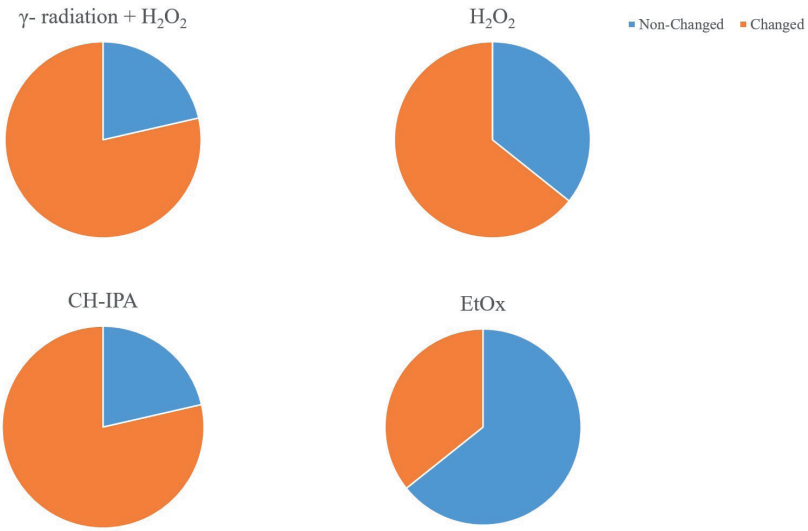

Figure 4 – Significant changes ($p \leq 0.05$) in biosensor performance parameters following each of the sterilization procedures, when compared with its performance prior to sterilization.

Nonetheless, not all performance parameters have the same weight regarding o the ability of sterilized biosensor to selectively monitor changes in glucose when implanted. Therefore we have evaluated in more detail the impact of each sterilization method on biosensor performance.

5.3.2.1- Sterilization by Ethylene Oxide

Amongst all the tested sterilization procedures, ethylene oxide was the one that has induced the least changes in the calculated biosensor performance parameters (5/14). Exposure of the biosensors to EtOx resulted in a decrease in I_{Max} , SI I_{Max}, and glucose LRS when compared with values obtained in the pre-calibration. Additionally this procedure also induced in an increase in glucose LOD and in H_2O_2 LRS.

Since changes in H_2O_2 sensitivity are related to electrode active surface (Cordeiro et al. 2016; O'Neill et al. 2008; Rothwell et al. 2010), the increase in H_2O_2 LRS (1.91±0.18 vs 2,96 ± 0,64 nA/µM $p \leq 0.001$) points to an increase in surface availability. Interestingly the increase in surface availability did not induce a decrease in selectivity. No significant differences were observed in the H_2O_2 SC for UA nor for AA. Together, these data suggest a disruption of the inner Nafion membrane, thereby exposing a previously coated electrode surface.

The decrease of both I_{Max} and SI I_{Max}, as well as its derivative, glucose LRS points to significant changes in the activity of the enzymes immobilized in the hydrogel. Since I_{max} is related to V_{max} (O'Neill et al. 2008), a decrease in this parameter reflects a decrease in maximum velocity. The decrease in I_{Max} combined with no changes in enzyme affinity ($_{app}K_M$), fits well with the model for uncompetitive inhibition. Thus, it seems that EtOx may act as uncompetitive inhibitor for immobilized glucose oxidase. Although EtOx has been reported to inhibit enzyme activity (Matsuoka et al. 1990) it is the first time that its behavior as uncompetitive inhibitor is demonstrated. The decrease in selectivity for glucose, due to higher SC, is a consequence of lower sensitivity to H_2O_2, combined with higher surface availability.

Our data contradicts an earlier study where Nafion coated glucose biosensors were sterilized with EtOx that reported no changes in biosensor performance parameters. However, despite using the same permselective membrane, our biosensor design is significantly different than the one reported by Zhang (Zhang et al. 1991). Moreover, in the here presented biosensor sensitivities for H_2O_2 and glucose are much higher (≥ 30 fold) when compared with those reported by Zhang et al., allowing a more detailed detection of sterilization-induced modifications. Furthermore, the pioneer study by Zhang and colleagues included a low number of sterilized sensors (n=2).

It is evident that sterilization by EtOx affects biosensor performance negatively. Furthermore, we found that the EtOx has differential effects on different biosensor membranes. Nevertheless, biosensors sterilized with EtOx, were still very sensitive for glucose (LRS ≥30 nA/mM), indicating that they should be suitable to monitor physiologically relevant changes

in glucose *in vivo*.

5.3.2.2- Sterilization by H_2O_2

Biosensors sterilized with H_2O_2 induced changes in more than half (9 of 14) of the calculated performance parameters (Table 1 A and B). Interestingly, all parameters were glucose-related, suggesting that this method has no acute effect on the inner permselective membrane. Nonetheless it has a major effect on biosensor performance.

Our data show a decrease in I_{Max} and SI I_{Max}, as well as in the glucose LRS. Additionally, we observed that ${}_{app}K_M$ and SI ${}_{app}K_M$, LR and LOD all significantly increased. Changes in I_{Max} and LRS point to decrease in enzyme activity, most likely due to enzyme inactivation. Although exposure to H_2O_2 can lead to enzyme inactivation both in its soluble and immobilized form (Greenfield et al. 1975; von Woedtke et al. 1994; Wildberger et al. 1986), a study has reported no changes in glucose after H_2O_2 sterilization (von Woedtke et al. 2002). However, one must be aware the effect of H_2O_2 exposure can be device dependent (von Woedtke et al. 1994).

It is evident that sterilization by H_2O_2 has a strong negative impact on biosensor performance. Nonetheless, the values for key biosensor such as the LOD, LRS and LR, estimated after sterilization, indicate that the biosensors are still able to adequately monitor glucose levels *in vivo*.

5.3.2.3- Sterilization by γ- Radiation combined with H_2O_2

The use of γ –rad it is considered to be a more effective sterilization method when compared with the use of H_2O_2 alone. Although milder when compared with sterilization by γ –radiation alone (≥10 kGγ) it is has been reported that the use of this method induces significant changes in the performance of biosensors. Our data show that indeed, this method causes severe changes in biosensor performance. In fact the number of performance parameters altered after sterilization (11/14) is higher than sterilization by H_2O_2 alone. In addition to all the parameters that were altered by H_2O_2 sterilization, the use of γ-rad sterilization resulted in two additional parameters that were affected: the noise levels and LOD for H_2O_2.

As a consequence of the increase in noise levels, biosensors pretreated with γ radiation displayed a 2-fold increase in the LOD for both glucose and H_2O_2. Nonetheless, LOD the values are not much higher than for biosensors sterilized with other methods (≤1 μM for H_2O_2 and ≤50 μM for glucose), and still suitable for monitoring changes in glucose levels.

Although sterilization with γ-radiation seems harsher than other sterilization methods a closer look at biosensor performance provided some evidences on the contrary. Despite the finding that I_{Max} SI I_{Max} and LRS were lower than for the non- sterilized biosensors, they were 2-fold higher than in biosensors treated only with H_2O_2. Interestingly, we have not found any differences in the affinity constants, nor LR between biosensors with and without γ radiation treatment. Pre-treatment with γ rays irradiation may induced changes in either

hydrogel morphology and/or quaternary structure of immobilized enzymes. While irradiation even with low γ ray intensity (≤7kGy) results in severe denaturation of enzymes in aqueous solution, its effect on immobilized enzymes is, as far as we know, unknown.

Nonetheless, irradiation with γ rays is widely used in the assembly of hydrogels and other polymeric membranes (Inoue et al. 2006; Karadağ et al. 2005; Srinivas and Ramamurthi 2007). It is likely that exposure of these biosensors to γ radiation resulted in irreversible changes in the morphology of the hydrogel and/or outer membrane. Hence, limiting the diffusion of H_2O_2 limiting the access to the immobilized enzymes. Taken together, our data suggest that pre-treatment with γ radiation may prevent part of the damaging effects of the exposure to H_2O_2.

It is concluded that biosensors sterilized with γ radiation followed by H_2O_2 treatment, are as selective, as yet more sensitive than the ones sterilized only with H_2O_2. Moreover, they may be successfully applied for *in vivo* subcutaneous glucose biomonitoring.

5.3.2.4- Sterilization by Chlorohexidine and Isopropyl alcohol

Along with biosensors treated with γ radiation and H_2O_2, those exposed to CH- IPA were the ones with more changes in performance parameters. The use of chlorohexidine in combination with isopropyl alcohol as sterilizing agent resulted in significant changes in 11 out of 14 parameters analyzed. However, since changes in both glucose SC were greater when compared to the ones induced by γ –rad and H_2O_2 ($p \leq 0.001$ vs $p \leq 0.05$), we can assume that this was the most harmful of all sterilization methods tested.

This method was the only one to induce changes in H_2O_2 SC. Indeed, both the H_2O_2 SC for UA and AA have substantially increased (≥ 10 fold) when compared to pre-sterilization values. Intriguingly, we also observed a significant decrease in H_2O_2 LRS. Exposure of the biosensors to CH-IPA, even for a short period (30 min), clearly resulted in a massive modification of the inner permselective membrane. Apparently, it resulted in an almost complete disappearance of biosensor selectivity.

The massive effect of CH-IPA is probably due to IPA rather than to CH. IPA is an aliphatic alcohol, compounds that are well-known for their ability to act as organic solvents (Layek and Nandi 2013; Nagasawa et al. 2011). In fact, in its soluble form Nafion is stored solved in a mix of aliphatic alcohols. Immersion of the biosensors in a solution of CH-IPA may have induced a complete surface rearrangement, most likely with extensive implications for the hydrogel morphology.

All glucose-related performance parameters, except noise and LOD were significantly different than those estimated prior to sterilization. We observed a major decrease (nearly 10 fold) in I_{Max}. However, sterilized with CH-IPA resulted in a significant increase in SI I_{Max} which may be a consequence of the decrease in surface availability rather than changes in enzyme activity. As a matter of fact, the increase of SI I_{Max} points an higher enzyme activity when compared with values prior to the sterilization. Although enzyme activation due to

sterilization is highly unlikely, the impact of CH-IPA on hydrogel morphology may have allowed a better access of glucose to the immobilized enzymes. The decrease in $_{app}K_M$ and SI $_{app}K_M$, thus LR, further points to such condition.

It has been described that exposure of enzymes to CH (in concentrations as low as 0.01%) resulted in significant decreases in enzyme activity (Knuuttila and Söderling 1981). However, exposure of the immobilized enzymes to CH may not be main cause for the changes observed in glucose related parameters. Albeit some enzyme activity may have been lost due to exposure to CH, overall there was an unexpected increase in enzyme activity. It seems that the tremendous effect of exposure to IPA on biosensor surface properties, seemed to overcome the deleterious effect of CH on enzyme activity.

Nevertheless, the decrease in sensitivity, biosensor affinity, LR and especially selectivity indicate that sterilization of biosensors with CH-IPA may be the only method that results in biosensors unable to monitor subcutaneous glucose *in vivo*.

5.3.3- Post Calibration – Long term

It has been discussed that there is a lack of studies focused on the impact of sterilization on biosensor performance. Nonetheless a few dozen studies (von Woedtke et al. 2002) report the immediate effects of sterilization. However, none, except the inceptive study of Von Woedtke, goes beyond the acute effects of sterilization on biosensor performance. Although the authors focused solely on the evaluation a few performance parameters, they were the first to report the impact of long term sterilization. Therefore we have evaluated biosensors, weekly, up for 4 weeks after the sterilization was performed.

5.3.3.1 - Non-Sterilized biosensors

Non-sterilized biosensors, were used as control to assess the long term effects of sterilization on biosensor performance. These biosensors were calibrated, *in vitro*, every week, for 4 consecutive weeks. Consecutive evaluations of non-sterilized biosensors showed no significant differences in the profile of the consecutive calibrations (Figure 5). We did not observe differences in oxidation currents in response to consecutive additions of glucose.

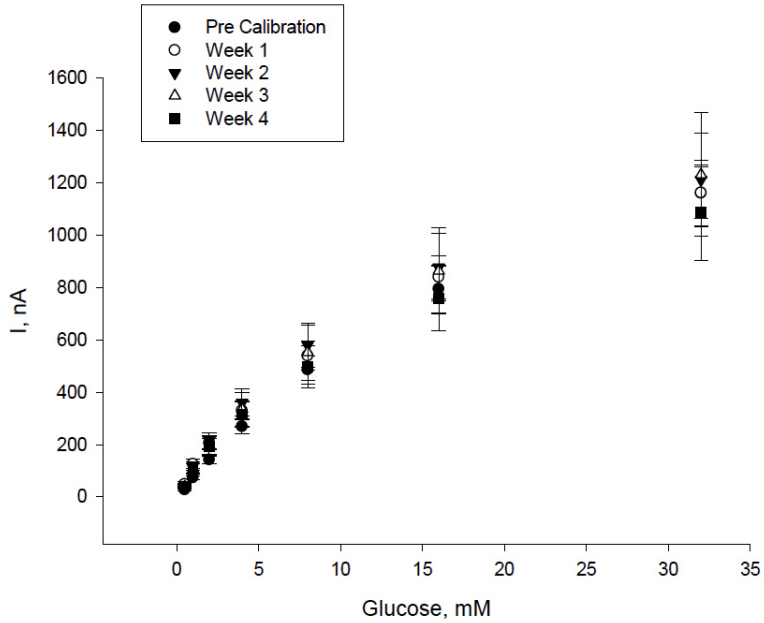

Figure 5 - *In vitro* calibration of the non-sterilized biosensors for four consecutive weeks. Data are mean±SEM.

However, fitting the data into the non-linear kinetic model revealed slight but significant differences in several biosensor performance parameters over the course of the 4 weeks following initial calibration (Fig S2), especially for glucose related parameters (Table 2 A and B).

Table 2- Biosensor performance parameters estimated from the weekly calibrations for non-sterilized biosensors. **A**-Glucose related parameters **B**-H_2O_2 related parameters. Data are mean ± SEM. * and ** denote a significant difference ($p \leq 0.05$ and $p \leq 0.001$) vs Pre Calibration.

A

		Pre-Calibration n=39	Post- Calibration	Week 1 n=8	Week 2 n=8	Week 3 n=8	Week 4 n=8
Glucose	Noise (nA)	0.08 ± 0.001	Not Available	0,06 ± 0,02	0,03 ± 0,01	0,13 ± 0,04	0,07 ± 0,01
	LOD (µM)	2,96 ± 0,05		1,65 ± 0, 07	0,92 ± 0,03	3,93 ± 0,13	2,37 ± 0,13
	$_{app}K_M$ (mM)	28.12 ± 0.78		17,93 ± 1,52 **	16,87 ± 1,09 **	20,82 ± 1,28 **	18,53 ± 1,96 **
	I_{Max} (nA)	2184.00 ± 34.94		1801 ± 76,19 **	1835 ± 58,33 **	2020 ± 65,37	1693 ± 90,62 **
	LRS(nA/mM)	77.67 ± 0.02		100.45 ± 4,25 **	108,77 ± 3,46 **	97,02 ± 3,14 **	91,37 ± 4,89 **
	LR (mM)	14.06 ± 0.39		8.97 ± 0,76 **	8,44 ± 0,55 **	10,41 ± 0,64 **	9,27 ± 0,98 **
	$SI_{app}K_M$	28.69 ± 0.67		17.17 ± 1,26 **	15,59 ± 0,80 **	19,45 ± 0,93 **	16,86 ± 1,53 **
	SI I_{Max}	1.14 ± 0.02		0,96 ± 0,03 **	0,96 ± 0,02 **	0,94 ± 0,02 **	0,77 ± 0,03 **
	SC AA	165.44 ± 30.28		215,07 ± 41,07	215,95 ± 30,64	240,58 ± 55,89	400, ± 99,3
	SC UA	128.27 ± 22.28		234,38 ± 39,22	294,19 ± 40,02	442,88 ± 125,2 **	416,32 ± 94,9 **
	R^2	≥ 0.99		≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99

B

		Pre-Calibration n=39	Post-Calibration	Week 1 n=8	Week 2 n=8	Week 3 n=8	Week 4 n=8
H_2O_2	LOD (µM)	0.23 ± 0.05	Not Available	0,06 ± 0,02	0,06 ± 0,01	0,34 ± 0,11	0,15 ± 0,04
	LRS (nA/(µM)	1.91 ± 0.18		1,80 ± 0,13	2,54 ± 0,24	1,93 ± 0,25	1,96 ± 0,16
	SC AA	3.76 ± 0.64		5,89 ± 1,12	4,71 ± 0,37	7,39 ± 1,38	9,12 ± 1,76 *
	SC UA	3.35 ± 0.47		6,84 ± 0,94	6,85 ± 0,51	11,02 ± 2,21	10,12 ± 1,68 *

No major changes were observed in the H_2O_2 related parameters, except for a slow gradual increase in both SC (AA and UA). Both parameters reached significantly higher values at week 4, when compared with pre calibrations values (3.76±0.64 vs 9.12±1.76 for AA and 3.35±0.47 vs 1012±1,68 , $p \leq 0.05$). Since no change in H_2O_2 LRS, thus in active surface, was observed this decrease in selectivity is most likely due to a reduction of the Nafion membrane. Although stored in the dark, at room temperature, the biosensors were in presence of O_2. This exposure may have induced changes in ion- exchange properties of Nafion membrane, reducing its selective properties only to size exclusion.

Despite an apparent similarity amongst all the calibrations, we observed significant differences in most glucose related parameters. Only the glucose LOD and the noise levels remained unchanged during the experiment.

We observed a significant decrease both ${}_{app}K_M$ and SI ${}_{app}K_M$, immediately at week 1 (28.12 ±0.78 and 28.69±0.67 vs 17,93±1,52 and 17,17±1,26, $p < 0.001$). Since the I_{Max} was only slightly affected, it is unlikely that this decrease may have been due to enzyme degradation/ inactivation. The hydration of the biosensor upon the initial calibration, combined with the dry storage, may have contributed to the changes affinity of the biosensor towards glucose. This sharp decrease in the affinity constants, resulted in a decrease in biosensor LR. However, none of these parameters were altered by any of the other calibrations, suggesting an irreversible yet stable alteration in the morphology of either the hydrogel morphology or the outer membrane.

Since no major changes in I_{Max} were observed, the decrease in LR implied a significant increase in LRS after 1 week, that remained fairly constant for the following 3 weeks.

Although at first glance it seemed that the biosensor performance remained unchanged throughout the whole experiment, we observed changes in glucose related performance parameters after week 1. No further changes, except of a gradual increase in H_2O_2 SC were observed throughout the remainder of the experiment. However, in a “real” application, the need for consecutive biosensor re-calibrations of would be residual. Nevertheless the study of non-sterilized biosensors provided some valuable insights to improve their storage conditions.

5.3.3.2- Sterilization by Ethylene oxide

Consecutive weekly evaluations of biosensors sterilized with EtOx resulted in significantly different calibration profiles. We observed (Figure 6) a significant decrease in the oxidation currents of the biosensors in response to high glucose concentrations (≥ 16 mM), from post calibration to week 1 and then to week 2 post-sterilization. No further differences in oxidation currents for any glucose level were observed, beyond week 2.

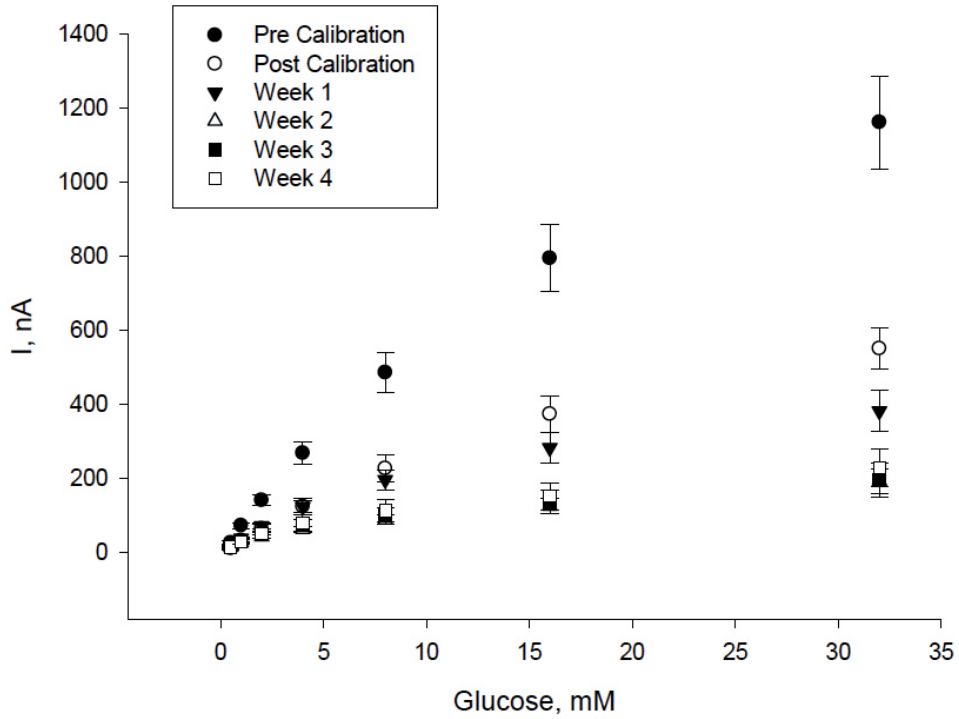

Figure 6 – Consecutive *In vitro* calibrations of biosensors sterilized with EtOx. Data are mean±SEM.

Additional gradual changes in biosensors performance (Figure S3) were seen when compared to those observed immediately after sterilization (Table 3 A and B).

Our data showed (Table 3 B) a gradual increase of H_2O_2 LRS from PreC to PostC and then to week 1. No further changes were observed after week 1. The gradual increase in H_2O_2 sensitivity, thus surface availability, had a negative impact on selectivity. We observed a gradual increase in SC for AA that reached statistical significance after week 1. No further changes were observed after week 1. Interestingly no significant increase was observed in the SC for UA. The lower oxidation currents by UA oxidation at the electrode surface (Cordeiro et al. 2016), when compared with AA may be the reason why the UA SC was not affected by the increase in electrode active surface. Collectively, these data suggest that the mild effect of EtOx on the inner membrane is amplified within the first week following sterilization.

Interestingly, the decrease in surface availability, with its negative impact on biosensor selectivity, did not induce an increase in the oxidation currents. Biosensors sterilized with EtOx displayed an additional decrease in I_{Max} and SI I_{Max} compared to that observed after sterilization from PC to week 1 and then to week 2. The decrease in I_{Max} along with a decrease in SI I_{Max}, suggest that the inhibitory effect of EtOx is long lasting and enhanced in the first 2 weeks after sterilization. Additionally we observed a decrease in both ${}_{app}K_M$ and SI ${}_{app}K_M$,

and its derivative LR, from PC to week 1. No further decrease was observed in any of these parameters until the end of the experiment. These data suggest that either hydrogel or outer membrane degradation due to exposure to EtOx may be responsible for the enhanced LR of these biosensors.

The changes observed in I_{Max} and $_{app}K_M$, resulted in an additional decrease in LRS from PC to week 1 and then to week 2, despite a significant decrease in LR from PC to week 1. Although the LR has decreased 2-fold and biosensor active surface increased, the sensitivity within this limited range has significantly decreased for the first two weeks post sterilization. This affected the LOD of the biosensors, which was almost 10 fold higher 4 weeks after sterilization when compared to values than prior to EtOx exposure. Consequently, the glucose SC for both AA and UA also suffered a gradual increase from PreC to week 2. As for all other performance parameters, no further changes were observed.

Overall, our data show that the deleterious effects of EtOx on biosensor performance are not confined to the procedure itself. These are amplified on long term storage when compared with its acute effects. While only 5 biosensor performance parameters are affected directly after sterilization, 11 are significantly different after 4 weeks storage.

Although the sterilization with EtOx resulted in a very pronounced decrease in biosensor performance, our data show that these biosensors still may be able to monitor *in vivo* glucose reliably. Although the selectivity, sensitivity and LR are severely impaired, the calculated LOD after 4 weeks is sufficient and the LRS although significantly lower, may be sufficient for *in vivo* glucose biomonitoring. However, the LR is lower than the maximum subcutaneous glucose levels expected for extreme hyperglycemia conditions. This may impair the ability of the sensors to monitor changes in glucose. However, recent advances in non-linear regression models for CGM (Facchinetti et al. 2014; Facchinetti et al. 2007; Lodwig and Heinemann 2003; O'Neill et al. 2008) combined with a recent evidences on the increase in biosensor LR (Cordeiro et al. 2015) even upon acute implantation ($\leq$ 8hrs), may enable accurate glucose biomonitoring with these biosensors.

Table 3- Biosensor performance parameters estimated from the weekly calibrations for biosensors sterilized with EtOx. **A**-Glucose related parameters **B**-H_2O_2 related parameters. Data are mean±SEM. * and ** denote a significant difference ($p \leq 0.05$ and $p \leq 0.001$) vs Pre Calibration.

A

		Pre-Calibration n=39	Post- Calibration n=6	Week 1 n=6	Week 2 n=6	Week 3 n=6	Week 4 n=6
Glucose	**Noise (nA)**	0.08 ± 0.001	0,11 ± 0,02	0,19 ± 0,04 *	0,10 ± 0,02	0,28 ± 0,10**	0,17 ± 0,05
	LOD (µM)	2,96 ± 0,05	9,15 ± 0,16 **	14,53 ± 0,37 **	16,53 ± 2,16 **	35,75 ± 3,49**	20,92 ± 2,24**
	$_{app}K_M$ (mM)	28.12 ± 0.78	29.40 ± 0,89	13.89 ± 0,77 **	14.74 ± 4,11 *	10.48 ± 2,43 **	12.60 ± 3,02 **
	I_{Max} (nA)	2184 ± 34.94	1059 ± 18,76 **	543 ± 13,88 **	269,7 ± 35,3 **	249,1 ± 24,32 **	305,3 ± 32,7 **
	LRS(nA/mM)	77.67 ± 0.02	36,02 ± 0,64 **	39,09 ± 0,10 **	18,30 ± 2,39 **	23,77 ± 2,32 **	24,23 ± 2,60 **
	LR (mM)	14.06 ± 0.39	14,70 ± 0,44	6,95 ± 0,39 **	7,37 ± 2,06 **	5,24 ± 1,22 **	6,30 ± 1,51 **
	SI $_{app}K_M$	28.69 ± 0.67	30,01 ± 0,78	18,09 ± 1,39 **	21,15 ± 6,32	15,21 ± 3,97 **	18,53 ± 5,06 **
	SI I_{Max}	1.14 ± 0.02	0,36 ± 0,01 **	0,07 ± 0,003 **	0,04 ± 0,007 **	0,03 ± 0,004 **	0,04 ± 0,005 **
	SC AA	165.44 ± 30.28	448,68 ± 150,22 *	3166,74 ± 456,83 **	7601,69 ± 1723,21 **	6708,59 ± 1362,65 **	7375,01 ± 1732,86**
	SC UA	128.27 ± 22.28	146,75 ± 41,18	518,38 ± 97,15	1240,62 ± 281,10 **	1261,08 ± 427,15**	1320,13 ± 374,56 **
	R^2	≥ 0.99	≥ 0.99	≥ 0.99	0,98	0,98	0.98

B

		Pre-Calibration n=39	Post-Calibration n=6	Week 1 n=6	Week 2 n=6	Week 3 n=6	Week 4 n=6
H_2O_2	**LOD (µM)**	0.23 ± 0.05	0,22 ± 0,06	0,13 ± 0,02	0,08 ± 0,009	0,13 ± 0,02	0,15 ± 0,06
	LRS (nA/(µM)	1.91 ± 0.18	2,96 ± 0,64	6,02 ± 0,50 **	5,23 ± 0,49 **	5,63 ± 0,65 **	6,12 ± 0,83 **
	SC AA	3.76 ± 0.64	6,58 ± 3,07	10,90 ± 1,51 **	12,05 ± 1,86 **	12,43 ± 2,20 **	12,94 ± 2,08 **
	SC UA	3.35 ± 0.47	2,47 ± 0,39	4,37 ± 0,82	5,20 ± 1,13	5,82 ± 1,99	5,75 ± 1,38

5.3.3.3- Sterilization by H_2O_2

We observed a significant increase in biosensor oxidation currents for high glucose levels (≥ 8 mM) at week 1 when compared with those observed immediately after sterilization (Figure 7). Nevertheless the oxidation currents were lower than observed prior to sterilization for most of the calibration range (≥ 1 mM).

After this increase, oxidation currents decreased once again after week 2 to values similar to those observed immediately after sterilization. No further changes in oxidation currents were observed in from week 2 to week 4 (Figure S4).

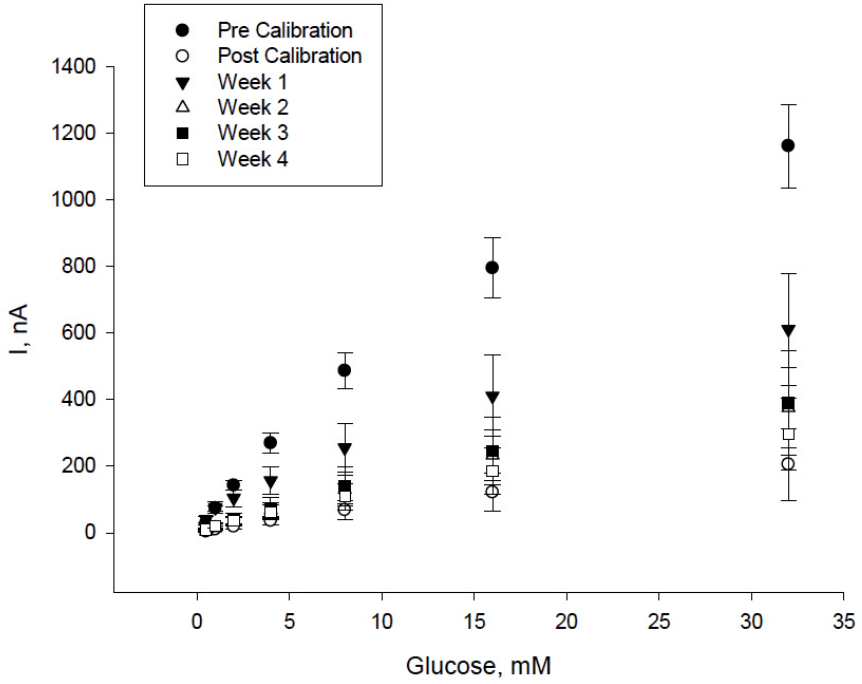

Figure 7 – Consecutive *In vitro* calibrations of biosensors sterilized with H_2O_2. Data are mean±SEM.

The changes in calibration profiles resulted in differences in several of the performance parameters, when compared with the values obtained pre and directly after post sterilization.

Our data (Table 4 B) show oscillations in the LRS for H_2O_2. While no changes where noticed after sterilization, H_2O_2 LRS at weeks 1 and 2 were higher when compared with PreC values. However, from week 2 on, we observed a progressive decrease to reach levels similar to those estimated prior to sterilization. Additionally, we observed a decrease in selectivity, thus increase in the H_2O_2 SC for both AA and UA from PostC to week 1, after which these

values remained unchanged. Data obtained by the PostC evaluation showed no effect of H_2O_2 exposure on the Nafion membrane. However, the data from consecutive evaluation shows a prolonged negative effect of this method on the properties of the permselective membrane.

Unfortunately, the effect of H_2O_2 sterilization was not confined to H_2O_2 related performance parameters. Our data show that I_{Max}, but not SI I_{Max} increased (nearly 2 fold) from PC to week 1 (621.6±19,28 vs 1033±107.3 nA, $p \leq 0.001$). This implies that the increase in I_{Max} is due to an increase in surface availability. Additionally, we observed a significant decrease in $_{app}K_M$ and SI $_{app}K_M$. to value similar to those observed prior to the sterilization. These changes implied significant changes in its derivative parameters, LR and LRS. After week 1 the LR was lower than observed in the PC (11,46±2,20 vs 32,46 ± 1,38 mM, $p \leq 0.001$). The increase in I_{Max}, combined with the decrease in LR resulted in a 5 fold increase in LRS (45,09±4,68 vs 9,57±0,30 nA/mM). In turn, the increase in LRS, coupled with the noise reduction resulted in decrease in glucose LOD to levels below 10 μM.

Table 4- Biosensor performance parameters estimated from the weekly calibrations for biosensors sterilized with H_2O_2. **A**-Glucose related parameters **B**-H^2O^2 related parameters. Data are mean±SEM. * and ** denote a significant difference ($p \leq 0.05$ and $p \leq 0.001$) vs Pre Calibration and # and ## denote a significant difference ($p \leq 0.05$ and $p \leq 0.001$) between PC and week 1 evaluation.

A

		Pre-Calibration n=39	Post- Calibration n=7	Week 1 n=7	Week 2 n=7	Week 3 n=7	Week 4 n=7
Glucose	**Noise (nA)**	0.08 ± 0.001	0,07 ± 0,01	0,01 ± 0,02 * ##	0,11 ± 0,01	0,09 ± 0,04	0,18 ± 0,04 **
	LOD (µM)	2,96 ± 0,05	21,83 ± 0,67**	6,63 ± 0,69* ##	18,52 ± 1,02 **	13,72 ± 0,18 **	33,04 ± 1,63 **
	$_{app}K_M$ (mM)	28.12 ± 0.78	64,92 ± 2,76 **	22,91 ± 4,39 ##	56,07 ± 4,39 **	47,34 ± 0,93 **	41,34 ± 3,15 **
	I_{Max} (nA)	2184 ± 34.94	621,6 ± 19,28	1033 ± 107,3** ##	1034 ± 56,96 **	965,9 ± 12,72 **	677 ± 33,48 **
	LRS (nA/mM)	77.67 ± 0.02	9,57 ± 0,30 **	45,09 ± 4,68 ** ##	18,44 ± 1,02 **	20,40 ± 0,27 **	16,3 ± 0,81 **
	LR (mM)	14.06 ± 0.39	32,46 ± 1,38 **	11,46 ± 2,20 ##	28,04 ± 2,19 **	23,67 ± 0,46 **	20,67 ± 1,58 **
	SI $_{app}K_M$	28.69 ± 0.67	59,39 ± 4,03 **	20,00 ± 4,82 ** ##	55,27 ± 3,02 **	48,92 ± 0,51**	40,43 ± 3,49 **
	SI I_{Max}	1.14 ± 0.02	0,23 ± 0,01 **	0,18 ± 0,02 ** #	0,23 ± 0,01 **	0,27 ± 0,002 **	0,23 ± 0,01 **
	SC AA	165.44 ± 30.28	2915,68 ± 1059,51 *	2998,99 ± 785,09 *	4492,60 ± 1643,22**	5110,43 ± 1735,73 **	4619,96 ± 1348,70 **
	SC UA	128.27 ± 22.28	960,43 ± 276,47	2612,92 ± 683,95	5884,67 ± 2228,92 **	6106,26 ± 2056,38 **	5647,41 ± 1662,19 **
	R^2	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99

B

		Pre-Calibration n=39	Post-Calibration n=6	Week 1 n=6	Week 2 n=6	Week 3 n=6	Week 4 n=6
H_2O_2	**LOD (µM)**	0.23 ± 0.05	0,23 ± 0,08	0,09 ± 0,02	0,12 ± 0,03	0,11 ± 0,04	0,28 ± 0,07
	LRS (nA/(µM)	1.91 ± 0.18	1,89 ± 0,40	4,71 ± 0,74 *	4,03 ± 0,63 *	3,05 ± 0,59	2,46 ± 0,35
	SC AA	3.76 ± 0.64	2,23 ± 1,13	8,81 ± 2,25	12,34 ± 4,01 *	13,28 ± 3,41 *	13,68 ± 4,14 *
	SC UA	3.35 ± 0.47	3,80 ± 1,20	9,00 ± 2,27 *	7,94 ± 2,31	9,79 ± 2,72 *	9,73 ± 2,75 *

Collectively, our data suggest that the striking acute effect of H_2O_2 sterilization is somehow reversed 1 week after sterilization. It is possible that a significant amount of H_2O_2 molecules have been entrapped within the hydrogel, or between the hydrogel and outer membrane wall. Indeed, the ability of some hydrogels to encapsulate/retain H_2O_2 for a significant amount of time has been reported (Hoare and Kohane 2008; Sudur and Orbey 2015; Sudur et al. 2015; Trusek-Holownia and Noworyta 2015). The partial recovery of biosensor performance one week after sterilization may be due to the fact that immediately after sterilization, a significant amount of H_2O_2 may have been entrapped within the enzymatic hydrogel. Its release upon calibration may be the reason why biosensor performance has increased form PC to week 1. The putative release of the entrapped H_2O_2, is a cause of concern. Significant amounts of H_2O_2 have been described to cause significant damage on the surrounding tissue of biosensor implantation sites. Nevertheless, biosensor performance hasn't reached the levels prior to its sterilization. Hence part of the decrease in biosensor performance after sterilization was due to enzyme inactivation by exposure to H_2O_2.

After the surprising increase in biosensor performance from PostC to week 1 we observed a slight decrease from week 1 to week 2, and no further noticeable changes thereafter. We have witnessed an increase in both SI $_{app}K_M$ and $_{app}K_M$, but no changes in either I_{Max} nor SI I_{Max}. This increase in $_{app}K_M$ is probably due to the irreversible enzyme inactivation by exposure to H_2O_2. In turn, the decrease in $_{app}K_M$ resulted in a decrease in LR and LRS.

As the SC is a function of analyte sensitivity, we observed a significant increase in glucose SC for both AA and UA when compared with the values estimated one week after sterilization. Here too, no further changes were observed beyond week 2.

Overall our data show that while some of the acute harmful effects of H_2O_2 exposure are partially reversed one week after sterilization, other become only noticeable at least one week after sterilization. Nevertheless the long term effects of biosensor exposure to H_2O_2 are quite complex and result in a significant decrease in biosensor performance. Regardless, biosensor performance estimated 4 weeks after sterilization indicate that they are still liable to detect glucose *in vivo*.

5.3.3.4- Sterilization by γ- radiation combined with H_2O_2

Consecutive calibrations of biosensors sterilized with γ-radiation in combination with H_2O_2 displayed different profile when compared with sterilization with H_2O_2 alone (Figure 8).

Following a significant decrease in oxidation currents immediately after sterilization, no further changes were observed, for any of the subsequent weekly calibrations.

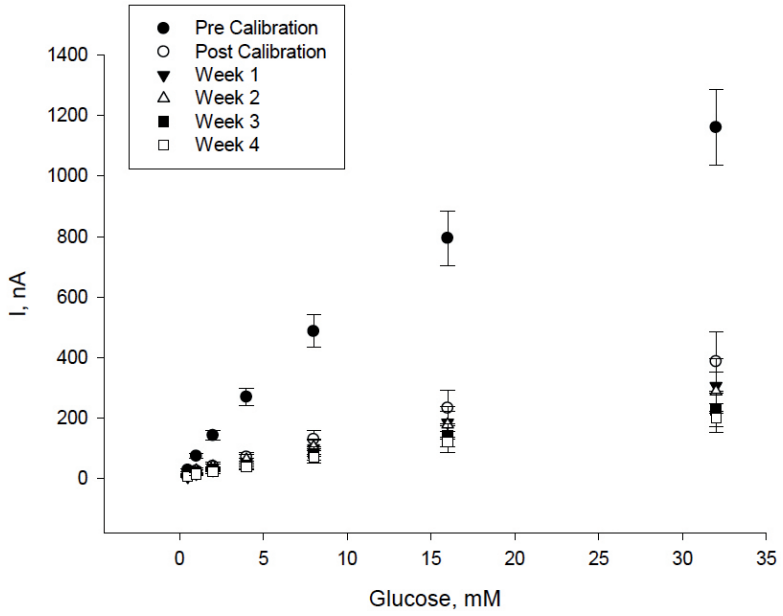

Figure 8 – Consecutive *In vitro* calibrations of biosensors sterilized with radiation combined with H_2O_2. Data are mean±SEM.

We did not observed major changes in H_2O_2 related parameters besides the increase in SC for both AA and UA (Table 5-B). The values of H_2O_2 SC 4 weeks and its gradual increase are very similar to those obtained for non-sterilized biosensors, thus seem independent of the sterilization method. However, we did find slight, but additional differences in some of the glucose-related biosensors performance parameters, beyond the acute sterilization effects, especially after week 1 (Table 5A).

While no major changes were observed from PostC to week 1, some parameters were altered upon evaluation two weeks after sterilization. We observed a decrease in both IMax and $_{app}K_M$ that would imply an increase in biosensor affinity along with a decrease enzyme activity. This may be due to a decrease in the amount of enzymes active within the hydrogel. The decrease in $_{app}K_M$, implied a decrease in LR, with no impact on biosensor sensitivity within its LR.

Although selectivity seemed to be gradually decreasing after biosensor sterilization, it only reached significantly different values from PreC, two weeks after sterilization. These values remained unchanged throughout the rest of the experiment. This might be a consequence of the slow degradation of the inner permselective membrane, enhanced by the decrease in biosensor sensitivity to glucose.

Additional pre-treatment with γ radiation resulted in a more stable, yet less effective

behavior than for biosensors sterilized only with H_2O_2. The morphologic changes induced by preliminary γ irradiation may have hindered the complete extent of the effect of H_2O_2 exposure on biosensor performance. However, in the long run no major changes were observed between biosensor sterilized H_2O_2 with and without γ-radiation. It is concluded that sensors sterilized with H_2O_2 combined with γ-radiation are suitable for *in vivo* biomonitoring of subcutaneous glucose.

Table 5- Biosensor performance parameters estimated from the weekly calibrations for biosensors sterilized with γ radiation and H_2O_2. **A-**Glucose related parameters **B-**H_2O_2 related parameters. Data are mean ± SEM. * and ** denote a significant difference ($p \leq 0.05$ and $p \leq 0.001$) vs Pre Calibration.

A

		Pre-Calibration n=39	Post- Calibration n=8	Week 1 n=8	Week 2 n=8	Week 3 n=8	Week 4 n=8
Glucose	**Noise (nA)**	0.08 ± 0.001	0,27 ± 0,06 **	0,19 ± 0,03 *	0,22 ± 0,06 **	0,10 ± 0,02	0,12 ± 0,02
	LOD (µM)	2,96 ± 0,05	43,75 ± 2,77 **	40,28 ± 1,73 **	41,75 ± 4,70 **	24,89 ± 2,48 **	35,23 ± 2,71 **
	$_{app}K_M$ (mM)	28.12 ± 0.78	58,42 ± 5,21 **	67,33 ± 3,95 **	39,66 ± 6,97 *	40,85 ± 6,29 *	49,32 ± 5,58 **
	I_{Max} (nA)	2184 ± 34.94	1091 ± 69,24 **	950,6 ± 41 **	637,7 ± 71,81 **	514,7 ± 51,26 **	503,7 ± 38,82 **
	LRS(nA/mM)	77.67 ± 0.02	18,68 ± 1,19 **	14,12 ± 0,61 **	16,08 ± 1,81 **	12,60 ± 1,25 **	10,21 ± 0,79 **
	LR (mM)	14.06 ± 0.39	29,21 ± 2,61 **	33,67 ± 1,97 **	19,83 ± 3,48	20,43 ± 3,15 *	24,66 ± 2,79 **
	SI $_{app}K_M$	28.69 ± 0.67	54,52 ± 4,53 **	62,98 ± 3,02 **	40,52 ± 6,53	42,28 ± 8,04 *	56,65 ± 7,65 **
	SI I_{Max}	1.14 ± 0.02	0,43 ± 0,03 **	0,38 ± 0,01 **	0,3 ± 0,03 **	0,18 ± 0,02 **	0,26 ± 0,02 **
	SC AA	165.44 ± 30.28	1561,41 ± 405,57	1794,06 ± 403,32	2661,01 ± 779,33 **	1716,13 ± 1280,93	3722,34 ± 931,18 **
	SC UA	128.27 ± 22.28	960,24 ± 243,03	1466,81 ± 311,31	3936,77 ± 1277,94 **	1990,31 ± 1790,69	4584,17 ± 1139,18**
	R^2	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99

B

		Pre-Calibration n=39	Post- Calibration n=8	Week 1 n=8	Week 2 n=8	Week 3 n=8	Week 4 n=8
H_2O_2	**LOD (µM)**	0.23 ± 0.05	0,59 ± 0,12 *	0,39 ± 0,05	0,47 ± 0,11	0,19 ± 0,03	0,30 ± 0,07
	LRS (nA/(µM)	1.91 ± 0.18	2,39 ± 0,19	2,39 ± 0,14	2,25 ± 0,15	2,91 ± 0,26	2,47 ± 0,07
	SC AA	3.76 ± 0.64	6,71 ± 1,01	5,93 ± 0,93	9,17 ± 1,34 *	6,97 ± 1,04	9,39 ± 1,48 **
	SC UA	3.35 ± 0.47	4,84 ± 0,81	5,89 ± 1,26	16,02 ± 3,60**	10,58 ± 1,99 **	13,45 ± 2,34 **

5.3.3.5- Sterilization by Chlorohexidine and Isopropyl alcohol

After a sharp decrease immediately after sterilization with chlorohexidine and isopropyl alcohol, we did not observe any additional differences glucose oxidation currents (Figure 9).

Subsequently, no further changes were observed either in H_2O_2- nor glucose-related parameters (Table 6 A and B), in addition to those observed immediately observed after sterilization. The changes induced to the morphology of the biosensors by its exposure CH-IPA seemed to be irreversible and unaffected even 4 weeks after sterilization. Due to its poor performance, biosensors sterilized with CH-IPA are the only ones that won't be suitable to reliably monitor glucose *in vivo*.

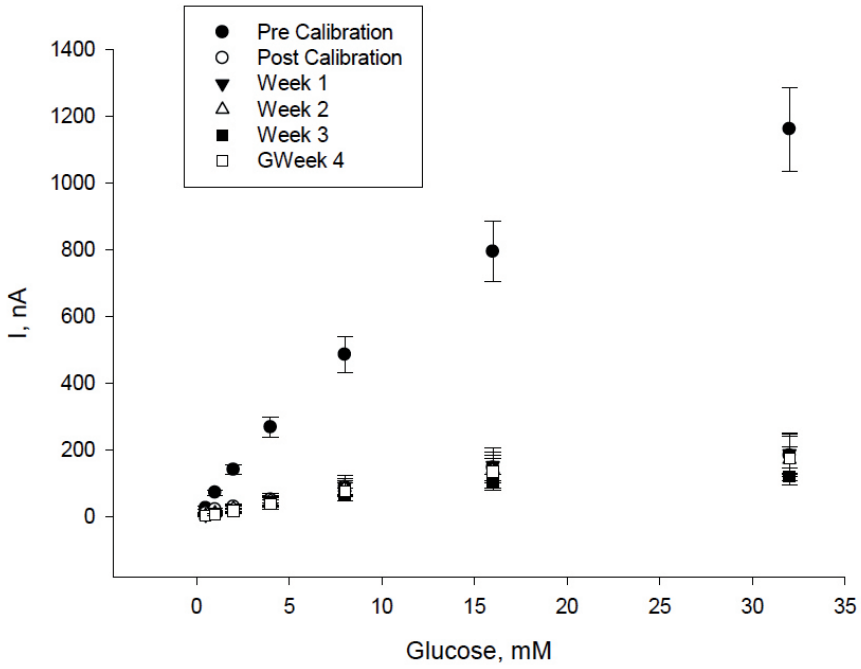

Figure 9 – Consecutive *In vitro* calibrations of biosensors sterilized with CH-IPA. Data are mean±SEM

Table 6- Biosensor performance parameters estimated from the weekly calibrations for biosensors sterilized with CH-IPA. **A**-Glucose related parameters **B**-H_2O_2 related parameters. Data are mean±SEM. * and ** denote a significant difference ($p \leq 0.05$ and $p \leq 0.001$) vs Pre Calibration.

A		**Pre-Calibration n=39**	**Post-Calibration n=8**	**Week 1 n=8**	**Week 2 n=8**	**Week 3 n=8**
Glucose	**Noise (nA)**	0.08 ± 0.001	0,03 ± 0,01 **	0,04 ± 0,01 * *	0,05 ± 0,01 *	0,08 ± 0,01
	LOD (µM)	2,96 ± 0,05	4,44 ± 0,29	6,98 ± 0,51	9,40 ± 0,69	16,57 ± 1,11
	$_{app}K_M$ (mM)	28.12 ± 0.78	15,56 ± 2,1 **	17,56 ± 2,60 **	15,8 ± 2,41 **	12,07 ± 1,84
	I_{Max} (nA)	2184.00 ± 34.94	278,80 ± 17,99 **	302,50 ± 22,23 **	264,20 ± 19,34 **	172.00 ± 11,56
	LRS(nA/mM)	77.67 ± 0.02	17,92 ± 1,16 **	17,23 ± 1,27 **	16,72 ± 1,22 **	14,25 ± 0,96
	LR (mM)	14.06 ± 0.39	7,78 ± 1,05 **	8,78 ± 1,30 **	7,90 ± 1,20 **	6,04 ± 0,92 *
	SI $_{app}K_M$	28.69 ± 0.67	12,27 ± 1,86 **	16,87 ± 2,51 **	16,4 ± 2,93 **	15,24 ± 2.16
	SI I_{Max}	1.14 ± 0.02	1,35 ± 0,09 *	1,00 ± 0,07	1,18 ± 0,10	0,83 ± 0,06 *
	SC AA	165.44 ± 30.28	2061,36 ± 869,07	1624,88 ± 625,41	2124,86 ± 987,99	2510,62 ± 1514
	SC UA	128.27 ± 22.28	1227,44 ± 519,92	1607,99 ± 650,73	1857,22 ± 898,91	2533,27 ± 1525
	R^2	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99	0.99

B		**Pre-Calibration n=39**	**Post- Calibration n=8**	**Week 1 n=8**	**Week 2 n=8**	**Week 3 n=8**
H_2O_2	**LOD (µM)**	0.23 ± 0.05	0,54 ± 0,21	0,45 ± 0,14	0,60 ± 0,14	1,39 ±0,71 *
	LRS (nA/(µM)	1.91 ± 0.18	0,25 ± 0,09 **	0,36 ± 0,14 **	0,29 ± 0,07 **	0,28 ± 0,06 **
	SC AA	3.76 ± 0.64	34,99 ± 10,24 **	18,78 ± 4,60 **	29,15 ± 7,15 **	24,04 ± 4,62 **
	SC UA	3.35 ± 0.47	50,61 ± 15,19 **	44,29 ± 11,54 **	60,61 ± 16,27**	59,99 ± 12,87 **

5.3.3.6- A summary of the long term effects of biosensor sterilization

The detailed characterization of the electrochemical properties of the biosensor allowed us to obtain a better understanding of on the effects of sterilization on biosensor performance. Based on the collected electroanalytical data, we performed a qualitative evaluation of the various sterilization procedures. In Table 7 the impact on the various sterilization protocols on the properties on both the permselective membrane and the enzymatic hydrogel is summarized.

Additionally, we deconstructed the effect of sterilization in overall biosensor performance. We qualitatively evaluated the effects of sterilization on the integrity of each membrane composing the biosensor molecular geometry.

Table 7 - Qualitative assessment of the long term effect (4 weeks) of each sterilization procedure on biosensor performance.

Sterilization Method	Permselective membrane	Enzymatic Hydrogel	Overall
EtOx	↓↓	↓↓↓	↓↓↓
H_2O_2	↓	↓↓	↓↓
γ- radiation + H_2O_2	↓	↓↓	↓↓
CH-IPA	↓↓↓	↓↓↓	↓↓↓

Legend- ↓, ↓↓, and ↓↓↓ denote the degrees of severity (respectively mild, severe and extreme) of the effects of each sterilization method on the performance on either overall biosensor or individual membrane performance.

Collectively our data suggest that both methods that use H_2O_2, had a mild effect on the integrity of permselective membrane. Although we observed a decrease in selectivity (up to 4 fold increase in the SC) towards H_2O_2, the LOD and LRS for H_2O_2 were not different than observed prior to sterilization. Sterilization by EtOx was considered to severely affect permselective membranes. Besides a decrease in selectivity, also the LRS for H_2O_2 has been significantly affected (3 fold increase). But no method affected the permselective membranes as deeply as sterilization by CH-IPA. After four weeks storage biosensors sterilized with CH-IPA were lost almost entirely its selectivity. Additionally the LRS for H_2O_2 was nearly 10-fold lower than initially.

Despite the significant impact of each method on the integrity of the permselective membrane, the effect of sterilization was overall, much more adverse on the properties of the enzymatic hydrogel. The degree of severity of the sterilization impact on hydrogel properties

was higher or similar (for CH-IPA) than the effect on the permselective membrane. Once again, biosensors sterilized with CH-IPA and EtOx were most affected but the negative effects of methods that included exposure to H_2O_2 were also evident.
Taken together it is concluded that exposure to H_2O_2 (alone or in combination with γ radiation) was less harmful when compared to treatment with EtOX or CH-IPA. The efficacy of H_2O_2 and the additional level of sterilization provided by exposure to γ radiation, recommends this procedure as method of choice.

Whilst sterilization of biosensors for *in vivo* implantation is possible, easing the sterilization requirements may be the key to a wider application of biosensors for in vivo biomonitoring.

5.3.4- Can we sterilize implantable amperometric enzyme-based biosensors?

All of the sterilization methods tested induced significant changes in biosensor performance immediately after sterilization. However the extent of the (adverse) impact was method dependent. While most of the deleterious effects can be observed immediately after each procedure, independently of the method. Nonetheless, for most methods, the negative effects increased up to two weeks after sterilization.

Since there are no specific guidelines on how to classify the efficacy of methods for biosensor sterilization, we performed, based on the electroanalytical data, a qualitative analysis. Based on those criteria, we concluded that sterilization with H_2O_2 in combination with gamma-radiation seems to be the best and CH-IPA the worst, both directly after sterilization or after a 4 weeks storage period.

Although several parameters are markedly affected by all sterilization methods, the superior performance of the presented biosensor enables the use *in vivo* of all investigated sterilization methods apart from pretreatment with CH-IPA.

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